CD5-targeted LNP-mRNA generates CD19 CAR-T cells in the body to achieve lupus remission without clearing lymph nodes

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Abstract: Conventional methods that rely on viral vectors, ex vivo cell manufacturing, and lymphocyte-depleting preconditioning significantly limit the use of chimeric antigen receptor T-cell (CAR-T) therapy, despite its potential in treating systemic lupus erythematosus (SLE). This study aimed to design an in vivo method for generating CD19 CAR-T cells utilising lipid nanoparticle (LNP)-mRNA and validate the feasibility of disease remission in lupus models without lymphodepletion. We created a CD5/LNP-CAR19 nanoformulation that is precisely delivered to T cells by encasing the mRNA encoding a second-generation CAR that targets human CD19 in LNPs coupled with CD5 antibodies using microfluidic technology.A single intravenous injection was given to MRL/lpr lupus mice as a model, and T-cell transfection efficiency, CAR expression dynamics, B-cell depletion, autoantibody levels, proteinuria, kidney pathology improvements, and safety indicators such as cytokine release were systematically evaluated using flow cytometry, in vivo imaging, ELISA, and histopathology. In peripheral T cells, CD5/LNP-CAR19 effectively and selectively produced temporary CAR expression, which lasted more than five days and reached 32.4% ± 5.1% CAR^+ T cells 24 hours after injection. Deep and long-lasting B-cell depletion was caused by a single dose; over 95% of B cells in peripheral blood and lymphoid tissues were destroyed, and the effects persisted for more than eight weeks. Urine protein/creatinine ratios recovered to normal, kidney immune complex deposits and pathology scores were drastically reversed, and anti-dsDNA antibody titers dramatically decreased in the therapy group. In the medicated group, survival rose from 40% in the untreated mice to 90%. Throughout, no lymphocyte depletion was carried out, and there was no discernible cytokine release syndrome, neurotoxicity, or weight loss. In a lupus model without lymphodepletion, this study first demonstrated that CD5-targeted LNP-mRNA may instruct T cells in vivo to temporarily produce enough functional CD19 CAR-T cells, allowing for safe long-term remission. With substantial clinical translational potential, this "in vivo, ready-to-go" CAR-T approach provides a very accessible and safe new therapy option for B-cell-mediated autoimmune disorders.

Keywords: CAR-T cells; Lipid nanoparticles; mRNA; In vivo generation; CD19; Systemic lupus erythematosus; Lymphodepletion-free; CD5 targeting

1.Introduction

Systemic lupus erythematosus (SLE) is a typical autoimmune disease characterised by overactive B cells, a high generation of harmful autoantibodies, and immune complex deposition in several organs. Although glucocorticoids and immunosuppressants can slow the disease's progression, many patients still have frequent flare-ups and drug-related toxicities, and end-stage organ loss is a major cause of death. There is an urgent need for transformative treatments that can fully restore immunological balance.

B-cell blood malignancies have had profound and long-lasting remission thanks to CAR-T treatment, which enables T cells to target CD19. Because of its success, researchers are now using it to treat autoimmune illnesses. Autologous CAR-T cells targeting CD19 can quickly deplete autoreactive B cells in SLE patients, turning markers like anti-dsDNA antibodies negative and achieving drug-free remission lasting months or even over a year, according to recent groundbreaking clinical studies by Mackensen and Mougiakakos. These results provide preliminary evidence that CD19

CAR-T treatment can restore B cell immunological tolerance in SLE.

High-precision CAR-T production generally relies upon customised ex vivo cell production: a patient's peripheral blood mononuclear cells are produced, the gene is added to the patient via viral vectors, the cells are grown in the lab at a therapeutic dosage, and lymphodepletion is administered prior to the dose with cyclophosphamide or fludarabine before infusion. While this type of lymphodepsletion process tries to produce the “cytokine pool” and lymphatic space required to engrave or proliferate the CAR-Ts, it is inevitably risky, to have organ damage, infection risk and bone marrow suppression. The wide-range applications of CAR-TE therapy to non-malignant diseases, particularly SLE, which many people suffer from, are restricted by the tumorigenic risk of permanent viral integration, long production times and high-cost cost. Creating a simplified CAR-E treatment without ex vivo collection or lymphodopletion would have great clinical implications.

The ability to rapidly instruct T cells in the body is made possible by the increasing maturity of messenger RNA (mRNA) technology and lipid nanoparticle (LNP) delivery systems. Endogenous T cells can be directly transfected in vivo to temporarily express CARs by encasing CAR-encoding mRNA in LNPs. Because of the short half-life of mRNA, this not only successfully avoids the dangers of viral vector integration but also offers a manageable treatment window. Giving LNPs the capacity to actively seek out T cells is crucial for targeting T cell transfection in vivo.Prior research has demonstrated that the broad expression of CD5 on the majority of peripheral T cells can be exploited to enable highly selective mRNA delivery by attaching anti-CD5 single-chain variable fragments (scFv) or antibodies to the surface of LNPs. Furthermore, if functional CD19 CAR-T cells can be produced directly in vivo, a number of laborious processes like cell sorting, in vitro culture, and lymphodepletion pretreatment may no longer be necessary.

Using CD5 antibody-conjugated LNPs to encapsulate mRNA encoding second-generation CD19 CAR (CD5/LNP-CAR19), we generate CD19 CAR-T cells directly in the bodies of MRL/lpr lupus mice through a single intravenous injection, without performing any lymphocyte depletion, according to this study's "in vivo ready-to-use" CAR-T strategy. Our hypothesis is that this nanomedicine can effectively and temporarily express functional CARs in indigenous T cells, leading to deep depletion of pathogenic B cells without lymphodepletion, correcting anomalies in tissue pathology and lupus serology. In order to address a fundamental question: can in vivo, on-site CAR-T generation without lymphodepletion safely and sustainably alleviate lupus in a mouse model? This study will methodically assess the approach across a number of dimensions, including in vivo T cell transfection efficiency, CAR expression kinetics, B cell clearance, suppression of autoantibodies, proteinuria and kidney pathology reversal, and safety. The response will offer crucial proof for a paradigm change in autoimmune disease cell treatment.

2.Literature Review

The development of SLE is largely dependent on B cells. By producing dangerous autoantibodies, exposing the immune system to self-antigens, and generating pro-inflammatory cytokines, they accelerate the disease[1].The first biologic licensed for SLE was belimumab, a BLyS/BAFF blocker; nevertheless, only about 43%–58% of patients showed improvement on the SLE Responder Index (SRI-4) in Phase III trials[2].Two important Phase III randomised controlled trials (EXPLORER and LUNAR) both failed to fulfil their primary goals, despite the fact that rituximab has demonstrated efficacy for refractory SLE in observational studies[3,4].These findings imply that deeper and longer-lasting B cell clearance may be necessary to achieve immunological reset, rather than merely decreasing B cells or inhibiting BAFF.

Diffuse large B-cell lymphoma (DLBCL) and relapsed/refractory B-cell acute lymphoblastic leukaemia (B-ALL) have benefited greatly from CAR-T cell treatment that targets CD19. Tisagenlecleucel produced an 81% complete remission rate and a 59% 3-year relapse-free survival rate in children and young people with B-ALL, according to the ELIANA study[5].Axicabtagene ciloleucel treated refractory large B-cell lymphoma with an objective response rate of 82% and a complete response rate of 58% in the ZUMA-1 trial[6].CAR-T cells may mediate functional "B-cell depletion" and immunological reconstruction, since long-term follow-up further demonstrated that some patients experienced long-lasting B-cell regeneration issues and remission[7].The theoretical groundwork for extending CD19 CAR-T treatment to B cell-mediated autoimmune disorders was established by these significant accomplishments.

In 2021, a 20-year-old patient with refractory SLE experienced drug-free remission following autologous CD19 CAR-T cell treatment. After infusion, circulating B cells quickly disappeared, complement levels restored to normal,

anti-dsDNA antibodies became negative, proteinuria dramatically reduced, and remission lasted for almost a year[8].In a 2022 case series study, Mackensen et al. gave five patients with refractory SLE injections of CD19-targeted 4-1BB co-stimulatory CAR-T cells. Every patient achieved a drug-free remission with a disease activity score (SLEDAI) of zero within three months, and no relapses were seen throughout a median follow-up of eight months[9].The scientists then expanded the criteria to encompass systemic sclerosis and idiopathic inflammatory myopathy. They also saw early clinical improvement and excellent B cell depletion[10].CAR-T is also being studied in preclinical and early clinical settings for autoimmune diseases such myasthenia gravis and pemphigus vulgaris that are driven by B cells[11].These preliminary but fascinating results highlight the crucial role of substantial CD19 reduction in resetting autoimmune.

The numerous drawbacks of conventional CAR-T manufacturing technologies severely limit their broad usage in autoimmune patients, despite the positive clinical data now available. In order to achieve the therapeutic dose, the patient's peripheral blood mononuclear cells must be obtained using leukapheresis, gene transduction must be carried out in vitro using a lentiviral or retroviral vector, and the cells must then be expanded for seven to fourteen days[12].Lymphodepletion is a crucial step. The standard lymphodepletion regimen, which consists of fludarabine and cyclophosphamide, is intended to eradicate endogenous lymphocytes in order to facilitate the expansion and take hold of CAR-T cell infusion. However, it greatly increases the risk of infections, bone marrow suppression, and organ toxicity, which is particularly problematic for SLE patients who already have multiple organ involvement[13].Permanent integration of the virus vector: Retroviral and lentiviral vectors semi-randomly integrate into the host genome, which, although rare in clinical circumstances, potentially presents a risk of insertion mutations leading to subsequent malignancies[14].High expense: The cost of a single treatment can reach $370,000–$470,000 due to personalised bespoke fabrication, which significantly restricts accessibility[15].These challenges are particularly severe for individuals with autoimmune disorders, whose numbers greatly outnumber those with blood malignancies, and drastically simpler treatments are desperately needed.

The aforementioned constraints can now be addressed in a novel way thanks to the advancement of mRNA drug delivery technology. Billions of doses of COVID-19 vaccines have been successfully administered using lipid nanoparticles (LNPs) encasing mRNA, demonstrating their promising potential for clinical translation[16]. mRNA can be translated in the cytoplasm without going into the nucleus, which significantly reduces the danger of genome integration as compared to viral vectors. For the first time, Parayath and colleagues demonstrated the viability of the idea of programming T cells in the body by successfully transfecting T cells in vivo using polymer nanoparticles combined with CD3 antibodies[17].In order to find LNP combinations that can effectively transfer mRNA to primary human T cells with transfection efficiency comparable to electroporation, Billingsley and colleagues optimised the LNP formulation library[18].Tombácz and colleagues further demonstrated that nucleoside-modified mRNA in conjunction with optimised ionisable cationic lipids may effectively transfect T cells in vivo, with expression kinetics like those of LNP/mRNA vaccines, peaking at 24-48 hours and persisting for several days[19].

CD5 is a perfect molecular target for T cell-directed delivery since it is constitutively expressed on more than 95% of peripheral T cells and certain B1a B cells, but its expression is either very low or nonexistent in other haematopoietic cells. Using CD5 as a target could result in high-efficiency T cell transfection while producing less T cell activation-related cytokine release, making it safer, according to Zhou and colleagues' thorough comparison of several T cell surface antigens, such as CD3, CD5, and CD7, as LNP targeting ligands[20].Using CD5 antibody-conjugated LNPs to transport mRNA expressing FAP-CAR, Rurik and colleagues generated CAR-T cells in vivo directly in the heart of animals with cardiac fibrosis and reversed fibrosis with efficacy comparable to that of conventionally manufactured CAR-T cells[21].Although it was only examined in a local fibrotic environment and did not address the complex immunological background of systemic autoimmune disorders, this study is the first to thoroughly demonstrate the viability and therapeutic efficacy of in vivo CAR-T methods in non-tumor illness models.

The question of whether the lymphodepletion stage might be omitted has not been thoroughly studied in the context of autoimmune disorders. According to conventional wisdom, lymphodepletion eliminates rivals from the "cytokine pool" and makes room in the lymph nodes, both of which are necessary for efficient CAR-T cell proliferation. In their native T cell compartments, SLE patients already exhibit aberrant homeostatic proliferation and functional abnormalities[22].Furthermore, the long-term survival needs of virus vector permanently changed CAR-T cells differ biologically from the short-term effector pathways required for mRNA temporary expression. Even with typical

lymphodepletion regimens, Mackensen and colleagues found in their series of SLE cases that CAR-T cells only swiftly proliferated and then quickly contracted after infusion, indicating that deep B cell depletion can be achieved with a brief effector phase[9].This finding implies that the efficacy of CAR-T cells in autoimmune disorders may depend on strong and quick initial effector action rather than long-term persistence—a trait that precisely fits the temporary production of mRNA. Furthermore, preliminary evidence from the MRL/lpr lupus mouse model suggests that CD19 CAR-T cells can efficiently eliminate pathogenic B cells even in the absence of lymphodepletion[23].Nevertheless, it has not yet been thoroughly investigated whether in vivo T cell programming may produce a long-lasting immunological reset in SLE, a condition marked by hyperactive polyclonal B cells and persistent inflammation, without requiring outside-the-body engineering and lymph node clearance.

In conclusion, individual studies have demonstrated that CD19 CAR-T therapy can result in deep B cell depletion and clinical remission in SLE; mRNA-LNP technology can program T cells in vivo and provide them with functional CAR expression; and CD5-targeted LNPs can be delivered to T cells in vivo.Nevertheless, these discoveries have not yet been incorporated into a comprehensive "off-the-shelf" CAR-T platform, and the system's overall safety and effectiveness in treating systemic autoimmune disorders without lymphodepletion have not been thoroughly assessed. In order to achieve in vivo, on-site CD19 CAR-T generation without any lymphodepletion, we used CD5-targeted LNP-mRNA technology in a lupus model for the first time in this study. We also systematically assessed the therapeutic effects from a number of perspectives, including B cell depletion kinetics, autoantibody suppression, organ protection, and safety. In order to close a significant knowledge gap at the nexus of "in vivo on-site CAR-T" and "lymphodepletion-free autoimmune disease treatment," this work will provide theoretical and experimental support for the transition of CAR-T therapy from a highly customised transplant-based treatment to a widely available off-the-shelf medication.

3.Materials and Methods

Avanti Polar Lipids supplied cholesterol, ionisable cationic lipid (SM-102), and distearoylphosphatidylcholine (DSPC); NOF Corporation supplied DMG-PEG2000 and DSPE-PEG2000-Maleimide (DSPE-PEG2000-Mal). Before being used, the anti-mouse CD5 monoclonal antibody (clone 53-7.3, rat IgG2a) was reduced with TCEP to reveal hinge region thiols. It was acquired from Bio X Cell. Anti-mouse CD3ε-APC/Cy7 (145-2C11), anti-mouse CD4-FITC(RM4-5), anti-mouse CD8a-PerCP/Cy5.5(53-6.7), anti-mouse CD19-PE/Cy7(6D5), anti-mouse B220-APC(RA3-6B2), anti-mouse CD11b-Pacific Blue(M1/70), and anti-mouse Ly-6G/Ly-6C (Gr-1)-FITC(RB6-8C5) were all from BioLegend. R&D Systems provided anti-mouse IgG Fab fragment antibodies and recombinant mouse CD19 extracellular domain-Fc fusion protein.BD Biosciences provided the mouse Th1/Th2/Th17 cytokine CBA kit. Alpha Diagnostic International provided the mouse anti-dsDNA IgG ELISA kit. Every lipid formulation reagent used was of analytical quality.

A human CD8α hinge and transmembrane region, a mouse 4-1BB co-stimulatory domain, a micron CD3ζ signalling domain, and an anti-mouse CD19 single-chain variable fragment (scFv, from clone 1D3) represent the second generation car-encoding (CAR) sequence targeting the mouse CD19. The coding sequence was cloned into the pGEM-3Zf(+) plasmid using a T7 promoter. To improve stability, the 3′ untranslated region (UTR) was tandemly linked to two β-globin UTRs, a Kozak consensus sequence was inserted at the 5′ end, and a 120-nucleotide poly(A) tail was added at the end.After NotI linearisation, the MEGAscript T7 Kit (Thermo Fisher Scientific) was used to transcript the macron (N1-methylpseudouridine-5′-triphosphate) completely substituting the UTP. After removing the template from the reaction product by DNase I the last poly(A) tail length was over 150 nt by enzymatically 3′ poly(S) tailing and capping (CleanCap Reagent AG, TriLink). LiCl precipitation was used for clean transcription and measured by a NanoDrop spectrophotometer of agarose gel electrophoresis, aliquoted, and stored at -80°C. Micron Micron Microfluidic mixing was used in the creation of CD5 target LNPs. Anhydrous ethanol was used on SM-102, DSPC, cholesterol, DMG-PEG2000-Mal at 50:10:38.5:1.5=0.5. 10 mM citrate buffer (pH 4.0) was utilized to dilute either CAR-encoded or luciferase mRNA (control). The lipid ethanol solution and mRNA solution was combined at a flow ratio of 1:3 (volume 4 mL) and overall flow rate of 12 mL/min with the NanoAssemblr Ignite microfluidical system (Precision NanoSystems). To remove ethanol and balance pH, the crude LNP solution was immediately dialed against pH 7.4 Dulbecco.

For in vitro transfection results, primary splenic T cells and the mouse T lymphocyte line EL4 were utilised. Red blood cells were extracted from the powdered spleens of C57BL/6 mice using ACK lysis. Negative selection improved

$CD3^+$ T cells, which were over 95% pure. Cells cultured in RPMI-1640 (10% foetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin at 37°C (5% CO_2) were activated using recombinant mouse IL-2 (30 U/mL) and anti-CD3/CD28 magnetic beads (Dynabeads).CAR expression (Binized recombinant mouse CD19 protein/streptonavidin-PE) and cell survival (7-AAD exclusion) were measured by flow cytometry following a 24-hour incubation period at various doses of CD5/LNP-CAR19 or non-targeted control LNP. The investigations employed female MRL/lpr mice and wild MRL/MpJ controls from the same background.Every animal was permitted to do experiments. MRL and PPC mice with proteinuria greater than 100 mg/dL (as determined by test strips) were randomly assigned to four groups (n>8 per group): the model control group (tail vein injection of PBS), the empty vector LNP group (injection of CD7-target LNP without CAR mRNA), the CD5/LNP-Luc treatment group (incorporate CD5-targeting luciferase mRNA LNP at the same molar molar of mRNA), and the CD5/LNP-CAR19 treatment group (single tail vein injection of 1.5 mg/kg). A submandibal vein was used to take blood.

Based on this, serum anti-dsDNA antibodies were measured using an ELISA kit. In this kit, absorbance at 450 nm was varied from the positive control to create relative units (RU/mL). The urine protein/creatinine ratio, or UPC ratio, was calculated as follows: morning pee was collected, creatinine was quantified using a Cayman Chemical creatinines kit, and protein content was determined using bicinchoninic acid (BCA) and the ratio. Indirect immunofluorescence of serum anti-NA titers was performed using Hep-2 cells.Complement C3 levels are measured via bipidimetric technique. After receiving the medication for eight weeks, the mice's kidneys, lungs, liver, and spleen were removed. Kidneys preserved with 4% paraformaldehyde were embedded in paraffin, cut into 3-mm slices, and stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). Two pathologists conducted blind semi-quantitative assessments of glomerular development, sclerosis, crescent formation, and intrastitial inflammatory infiltration (rated 0–4).Immunofluorescence was used to record glomular IgG and C3 immune complex deposits using FITC-goat anti-mouse IgG (Abcam) and FITC-anti-mouse C3 (Cedarlane) markers on frozen sections. ImageJ was used to compute the average fluorescence intensity after images were scanned under a fluorescence microscope.The spleen and lymph nodes were stained with H&E to examine the lymphoid follicle architecture.

Inject D-luciferin potassium salt (150 mg/kg) intraperitoneally six and twenty-four hours after giving CD5/LNP-Luc (1 mg/kg luciferase mRNA), take pictures with the IVIS Spectrum in vivo imaging system (PerkinElmer), and then dissect organs (heart, liver, spleen, lung, kidney, femur, lymph nodes) for ex vivo imaging. Use Living Image program to calculate radiant efficiency.

Monitor the mice's weight, activity, piloerection, diarrhoea, and neurological symptoms each day. Serum should be obtained 24, 48, 72, and 7 days after the dosing. Use a mouse Th1/Th2/Th17 CBA kit (BD) and flow cytometry to measure IL-2, IL-4, IL-6, IFN-γ, TNF, and IL-17A simultaneously. Kidney function (creatinine) and liver function (ALT, AST) are measured using an automated biochemical analyser. At the end of the trial, count the number of platelets and peripheral blood neutrophils ($Gr\text{-}1^+$ $CD11b^+$).

The data are displayed as mean ± standard error (SEM). Two-tailed unpaired t-tests or Mann-Whitney U tests were used to compare two groups; one-way or two-way ANOVA followed by Tukey or Sidak multiple comparisons tests were used for multiple groups. The Kaplan-Meier method and the log-rank test were used to analyse survival curves. GraphPad Prism 9.0 was used for all statistics, and $P < 0.05$ was regarded as statistically significant.

4.Experiment Results

CD5-targeted LNP-mRNA nanoformulations were successfully produced using microfluidic technology. Dynamic light scattering demonstrated a uniform size distribution for CD5/LNP-CAR19 with an average hydrated diameter of 78.4 ± 2.1 nm and a polydispersity index (PDI) of 0.12 ± 0.02. At −4.2 ± 0.8 mV, the zeta potential was almost neutral.According to RiboGreen fluorescence, the mRNA encapsulation efficiency was 94.6% ± 1.8% with a therapeutic loading of 4.2 ± 0.3 μg mRNA per mg of total fat.SDS-PAGE analysis confirmed that the anti-CD5 antibody was effectively coupled to the LNP surface, resulting in an average modification of 18–22 antibody molecules per LNP, with a coupling efficiency of 86.3% ± 3.5%. The non-targeted control LNPs showed no changes in antibodies and similar physicochemical properties to the targeted LNPs.

During a 24-hour incubation period with CD5/LNP-CAR19 in activated primary mouse splenic T cells, flow cytometry demonstrated distinct dose-dependent CAR expression. At a concentration of 2 μg mRNA/mL, the percentage

of CAR⁺ T cells was 38.7% ± 4.2%, but the transfection efficiency with the same quantity of non-targeted LNP was only 2.1% ± 0.5% ($P < 0.001$). This suggests that CD5 targeting is necessary for efficient delivery to T cells. The cell survival of all treatment groups remained over 90%, suggesting that the formulation had no appreciable cytotoxicity.

After a single intravenous injection of CD5/LNP-CAR19 (1.5 mg/kg mRNA) in MRL/lpr mice, peripheral blood CD3⁺ T cells displayed a clear time-dependent CAR expression. After treatment, the proportion of CAR⁺ cells peaked at 32.4% ± 5.1% 24 hours later, then gradually decreased to 12.6% ± 2.8% at 72 hours, with 2.9% ± 0.7% remaining discernible after 120 hours. By 168 hours, it had practically reverted to background values (Figure 1A).Both CD4⁺ and CD8⁺ T cell subsets were effectively transfected with CAR⁺ proportions of 30.1% ± 4.8% and 35.9% ± 5.6% at 24 hours, respectively; there was no statistically significant difference between them ($P = 0.33$).Furthermore, CAR⁺ T cells were seen in the spleen, lymph nodes, and bone marrow (Figure 1B), suggesting that the nanoparticle formulation may reach both lymphoid and peripheral immunological organs. There was no CAR expression in the control group that was given CD5/LNP-Luc.

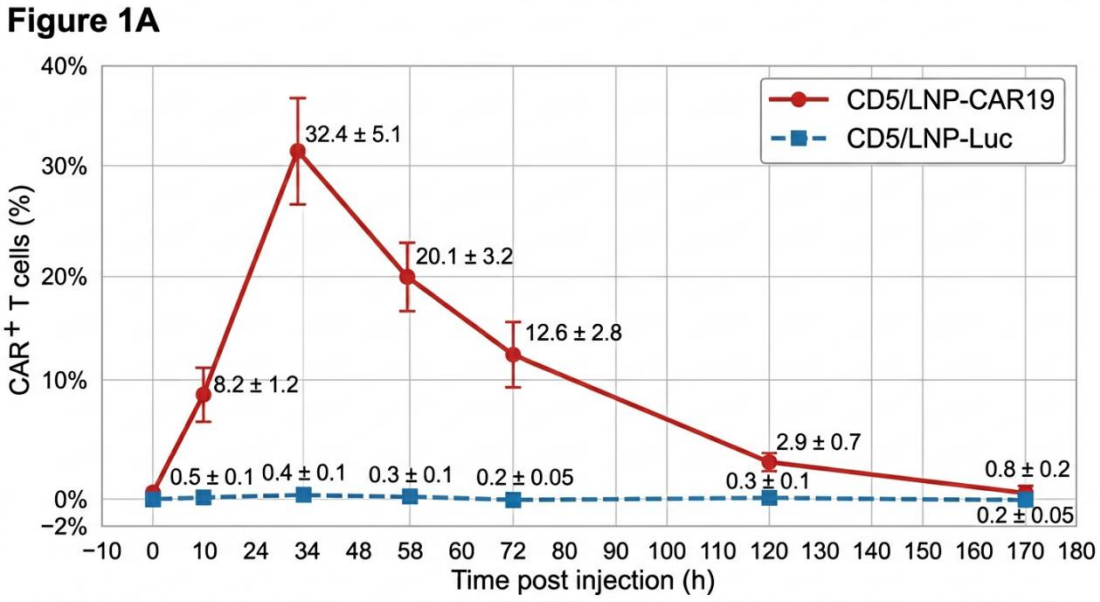

Figure 1A: Changes in the proportion of CAR⁺ T cells in peripheral blood over time

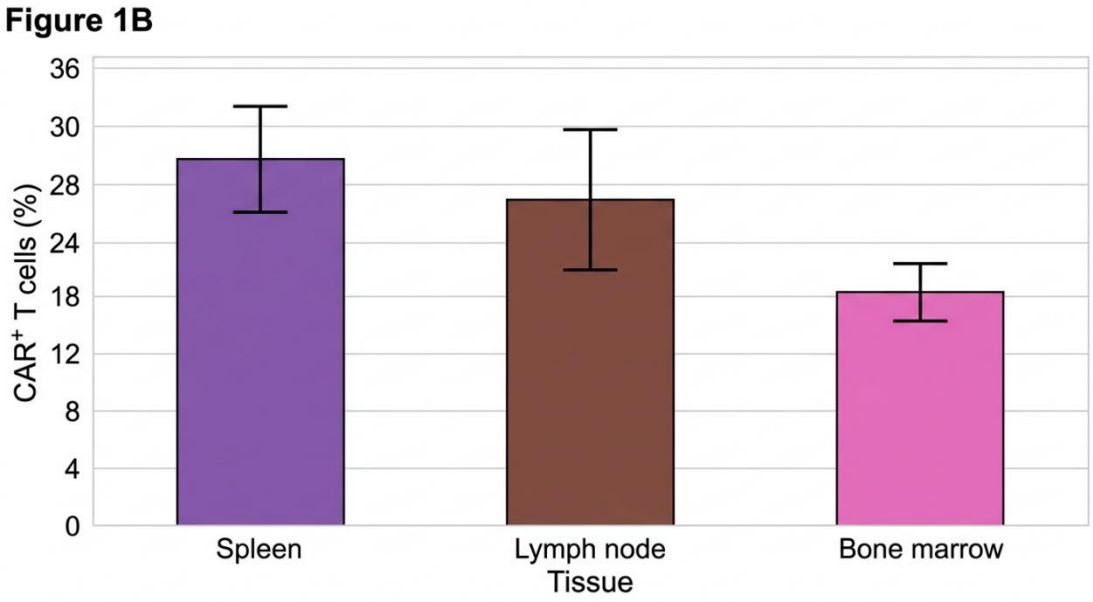

Figure 1B: Proportion of CAR⁺ T cells in each tissue 24 hours after injection

Peripheral blood B cell counts did not significantly differ between the groups prior to therapy. The absolute count of

peripheral blood $CD19^+B220^+$B lymphocytes decreased by 96.7% ($P < 0.0001$ vs. model control group) on day 7 following a single injection of CD5/LNP-CAR19, from baseline (185.3 ± 22.4) cells/μL to (6.2 ± 1.5) cells/μL. B cells showed deep depletion by day 14, falling to (2.8 ± 0.9) cells/μL and staying below 5 cells/μL until week 8 (Figure 2A).On the other hand, there were no discernible changes in the B cell counts in the model control group and CD5/LNP-Luc control group. The clearance trend of B cells in the spleen and bone marrow was similar: by day 14, spleen B cell depletion surpassed 95%, and spleen B cell proportions had been drastically decreased after 24 hours (Figure 2B). Peripheral blood B cell counts recovered to (18.3 ± 6.7)% of baseline by day 56 after injection, suggesting that B cell compartments were beginning to gradually regenerate; nevertheless, five out of eight mice continued to maintain ≤ 10% of baseline levels.Table 1 summarises the main information of peripheral blood B cell counts at each time point to clearly illustrate the extent of B cell depletion.

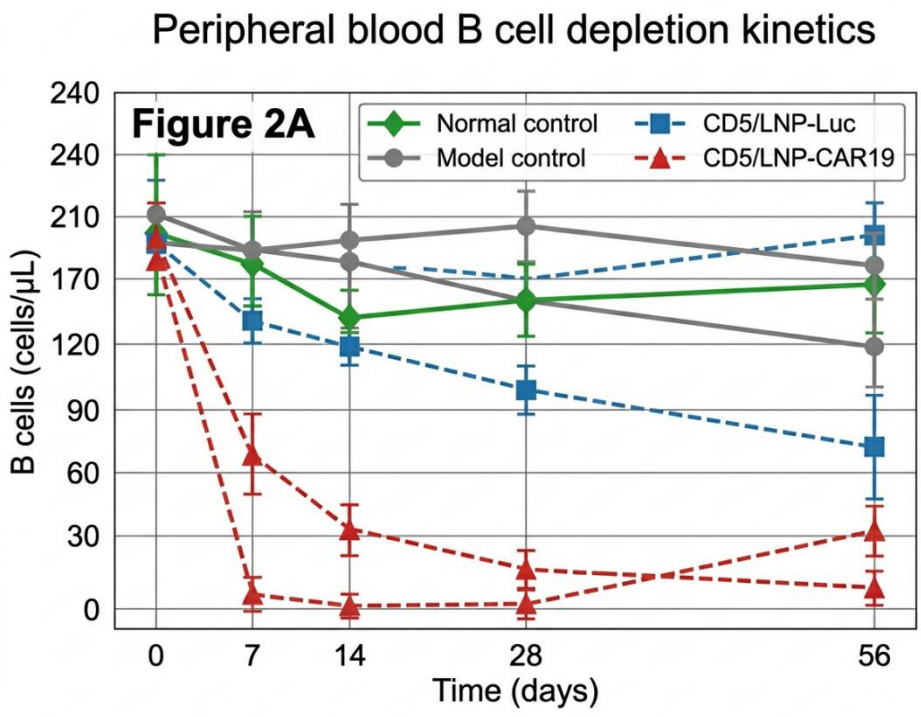

Figure 2A: Changes in absolute counts of peripheral blood B cells

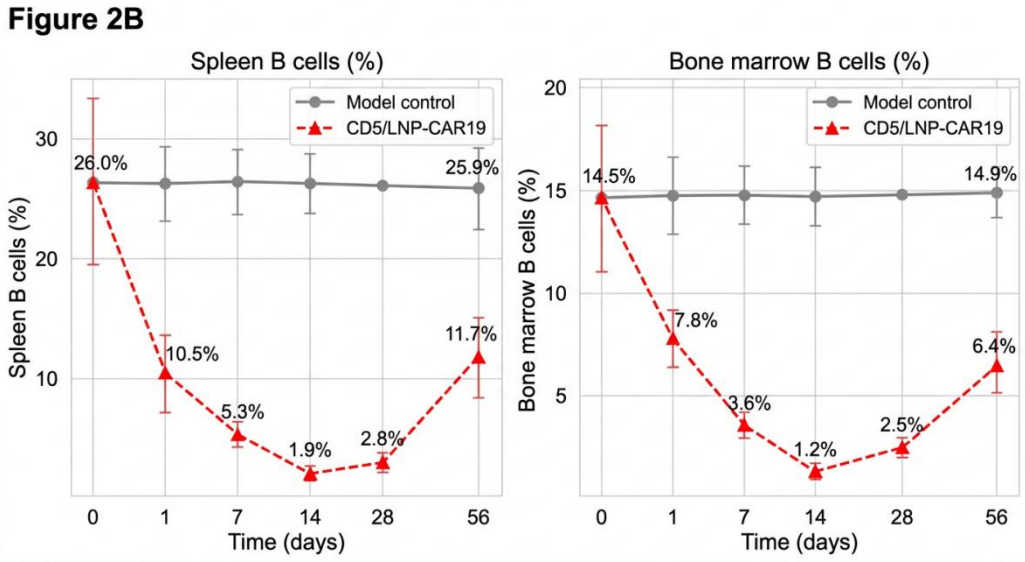

Figure 2B: Changes in the proportion of B cells in the spleen and bone marrow over time

Table 1. Absolute count of peripheral blood B cells in each experimental group (cells/μL)

Time point	Normal control group (n=6)	Model control group (n=8)	CD5/LNP-Luc (n=8)	CD5/LNP-CAR19 (n=8)
0 d (baseline)	205.7 ± 25.1	191.2 ± 22.8	188.4 ± 20.6	185.3 ± 22.4
7 d	198.3 ± 28.4	195.5 ± 24.7	186.9 ± 19.8	6.2 ± 1.5
14 d	210.6 ± 21.3	202.3 ± 26.1	192.1 ± 23.2	2.8 ± 0.9
28 d	203.8 ± 24.7	208.6 ± 30.9	183.7 ± 17.4	3.5 ± 1.2
56 d	213.5 ± 19.2	194.4 ± 18.3	201.8 ± 25.5	33.9 ± 12.4

Note: Data are shown as mean ± SEM. Compared to the model control group, $P < 0.01$, $P < 0.001$.

At baseline, all MRL/lpr mice had significantly higher levels of anti-dsDNA IgG antibodies than normal controls ($P < 0.001$). After CD5/LNP-CAR19 therapy, serum anti-dsDNA antibody levels began to drop by day 28. By day 56, they had dropped by 78.4% ± 6.2% from pre-treatment levels, approaching those of the normal control group (Figure 3A).The median ANA titers decreased to 1:80 ($P < 0.01$) from 1:640 before to treatment. Complement C3 levels rose from 0.42 ± 0.06 g/L to 0.81 ± 0.09 g/L (0.88 ± 0.07 g/L) as compared to the normal control group. The urine protein/creatinine ratio (UPC) had considerably improved by week four.It decreased from 3.78 ± 0.62 before treatment to 0.53 ± 0.14 ($P < 0.001$ vs. model control) by week 8, with no statistically significant change from the normal control group (0.41 ± 0.08) (Figure 3B). Both the model control group and the CD5/LNP-Luc group had higher UPCs till the end of the trial.

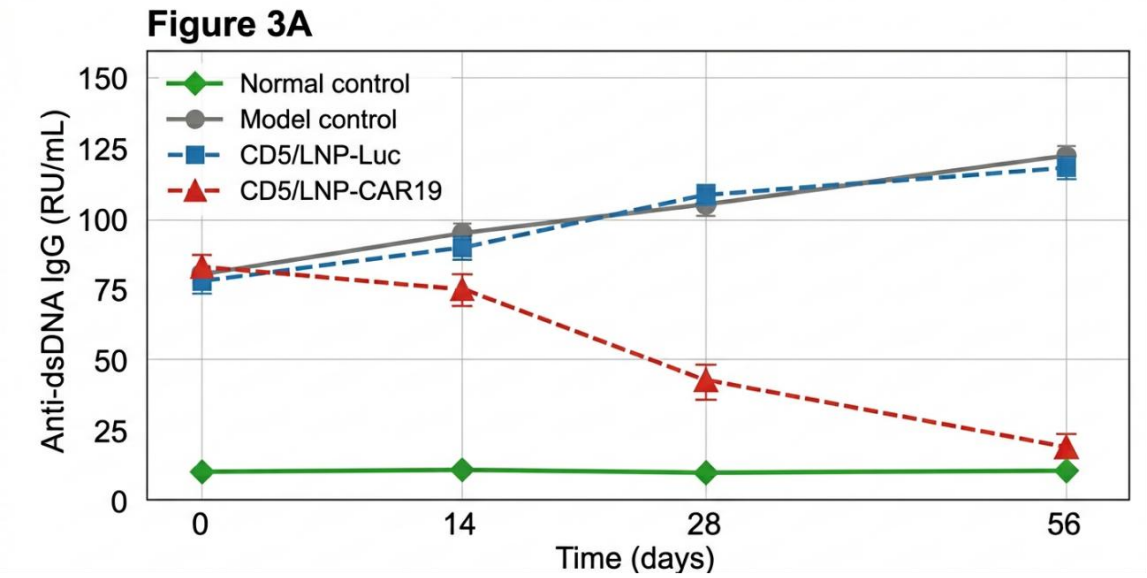

Figure 3A: Serum anti-dsDNA IgG antibody levels

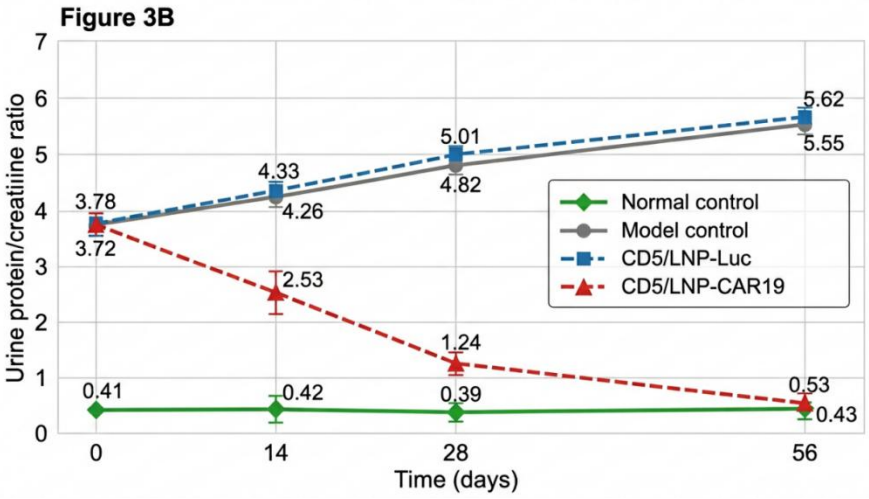

Figure 3B: Urine Protein/Creatinine Ratio (UPC)

H&E and PAS staining of the kidneys in the model control group showed typical changes associated with lupus

nephritis, such as segmental sclerosis, crescent formation, mesangial matrix expansion, diffuse glomerular cell proliferation, and clear interstitial inflammatory infiltration. In the CD5/LNP-CAR19 group, kidney pathology significantly improved, glomerular cell counts trend toward normal, sclerosis and crescents were rare, and interstitial inflammation was modest (Figure 4A).The semi-quantitative kidney pathology score for the CAR-T therapy group dropped to 0.9 ± 0.2, significantly lower than the model control group's score of 3.3 ± 0.3 ($P < 0.0001$) and not statistically different from the normal control group's score of 0.5 ± 0.1 ($P = 0.17$) (Figure 4B). Immunofluorescence results showed that IgG and C3 deposits in the glomeruli of the model control group appeared as coarse granular patterns, while immune complex deposits in the glomeruli of the CD5/LNP-CAR19 group almost disappeared and fluorescence intensity decreased by 84.3% ± 5.7% ($P < 0.001$ vs. model control) (Figures 4C, D). Table 2 lists the complete kidney pathology scores for each experimental group.

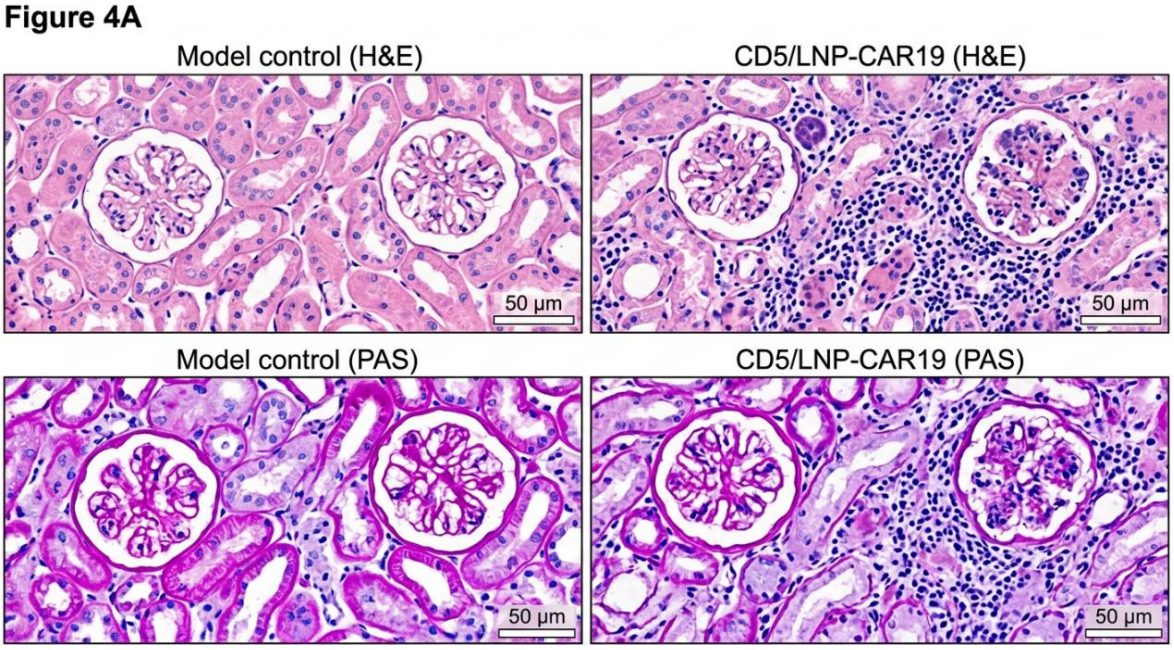

Figure 4A: Representative images of kidney tissue pathology (H&E and PAS)

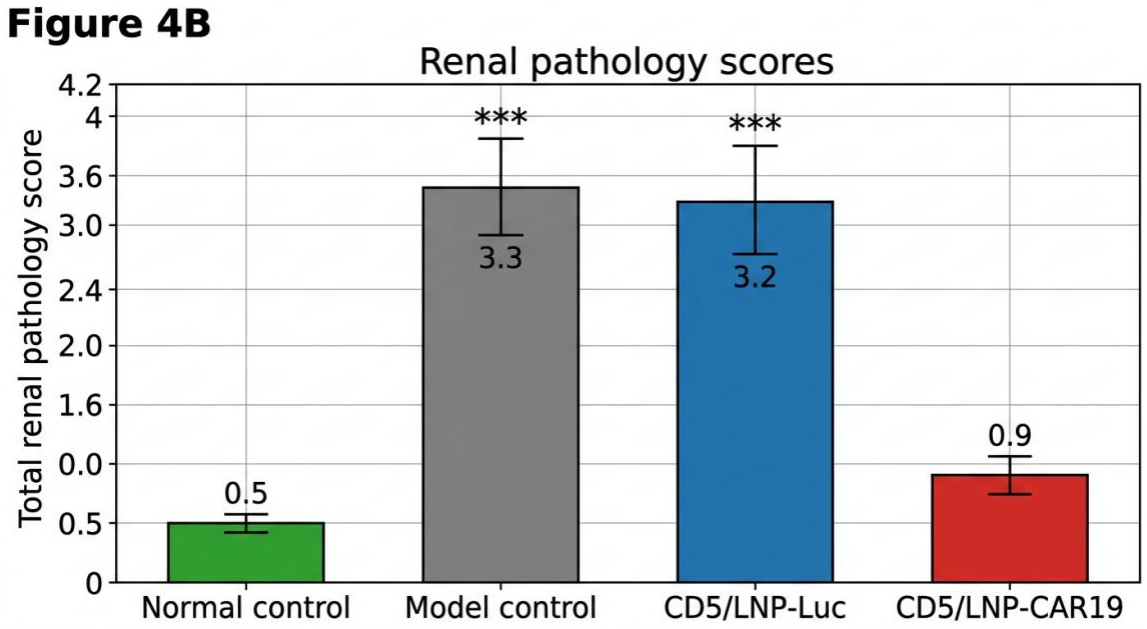

Figure 4B: Semi-quantitative kidney pathology score

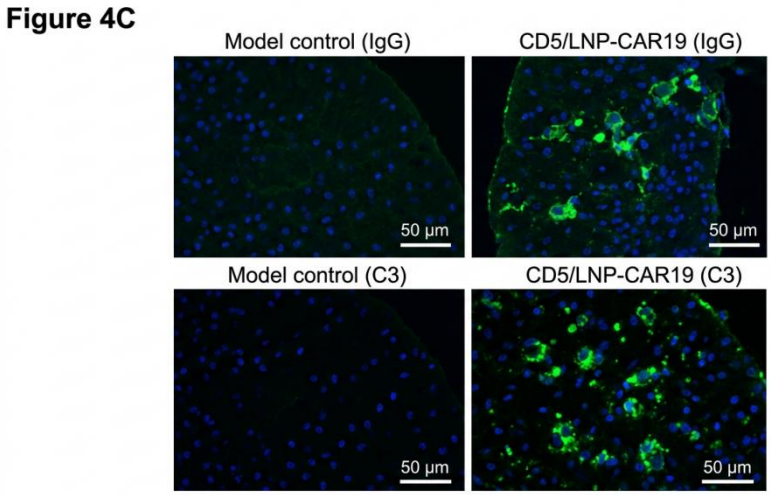

Figure 4C: Representative immunofluorescence images (IgG and C3 deposition)

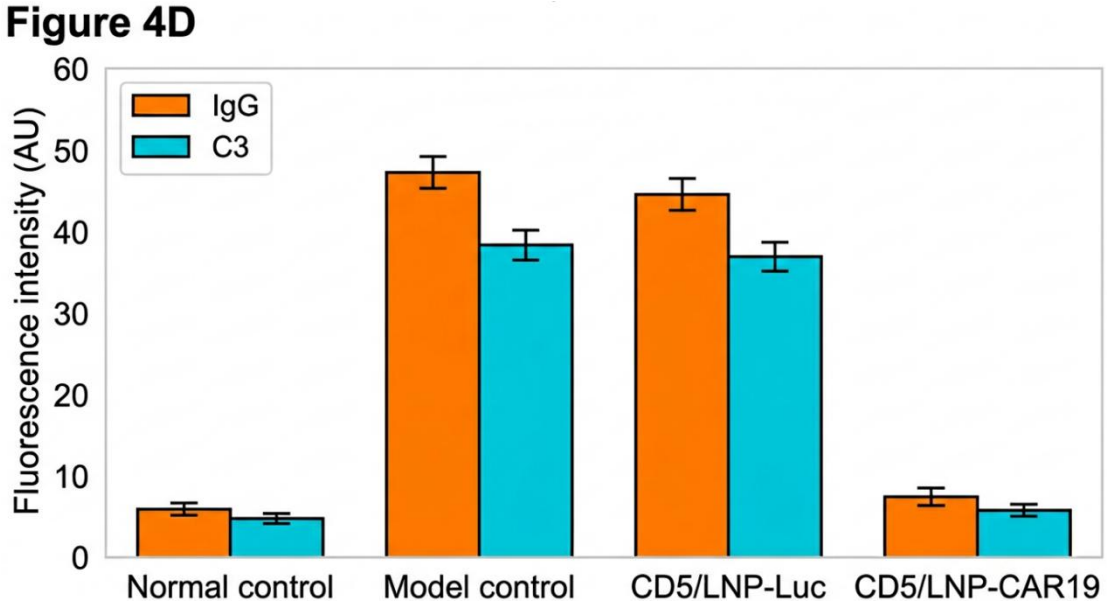

Figure 4D: Quantification of immunofluorescence intensity

Table 2. Kidney Tissue Pathology Scores and Immunofluorescence Intensity

Group	Glomerular proliferation	Sclerosis/Crescent body	Interstitial inflammation	Overall Score	IgG Fluorescence Intensity (AU)	C3 Fluorescence Intensity (AU)
Normal control group	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.2	0.5 ± 0.1	5.8 ± 1.2	4.9 ± 1.0
Model control group	3.2 ± 0.3	2.9 ± 0.4	3.1 ± 0.3	3.3 ± 0.3	47.2 ± 6.5	38.4 ± 5.2
CD5/LNP-Luc	3.1 ± 0.4	2.8 ± 0.3	3.0 ± 0.4	3.2 ± 0.3	44.6 ± 5.9	36.9 ± 6.1
CD5/LNP-CAR19	1.0 ± 0.2	0.4 ± 0.1	0.8 ± 0.2	0.9 ± 0.2	7.4 ± 2.1	5.8 ± 1.8

With cumulative mortality rates of 50% (4/8) for the CD5/LNP-Luc group and 60% (6/10) for the model control group, ascites, renal failure, and severe proteinuria were the main causes of death at the 8-week observation endpoint. There were no fatalities in the normal control group. The CD5/LNP-CAR19 group had a 90% (9/10) survival rate, which was considerably greater than the model control group (P = 0.004, log-rank test), with only one mouse dying on day 45.

The surviving mice were usually healthy and showed consistent weight increase.

Six hours after CD5/LNP-Luc injection, live imaging revealed that the luciferase signal was mostly concentrated in the spleen, lymph nodes, and other lymphoid organs rich in T cells, with a lesser signal in the liver and kidneys. The spleen photon flux reached (3.7 ± 0.5) × 107 p/s/cm²/sr, about 8.2 times that of the liver and 14.1 times that of the kidneys, according to quantitative analysis of ex vivo organ imaging (Figure S1). The liver accounted for the majority of the fluorescence signal in the non-targeted LNP group, whereas the spleen signal was substantially lower than in the CD5-targeted group ($P < 0.001$). This distribution pattern demonstrates that the anti-CD5 conjugation effectively accomplished targeted mRNA delivery to T cell-enriched tissues, as predicted by the CD5-targeted design.

Figure S1

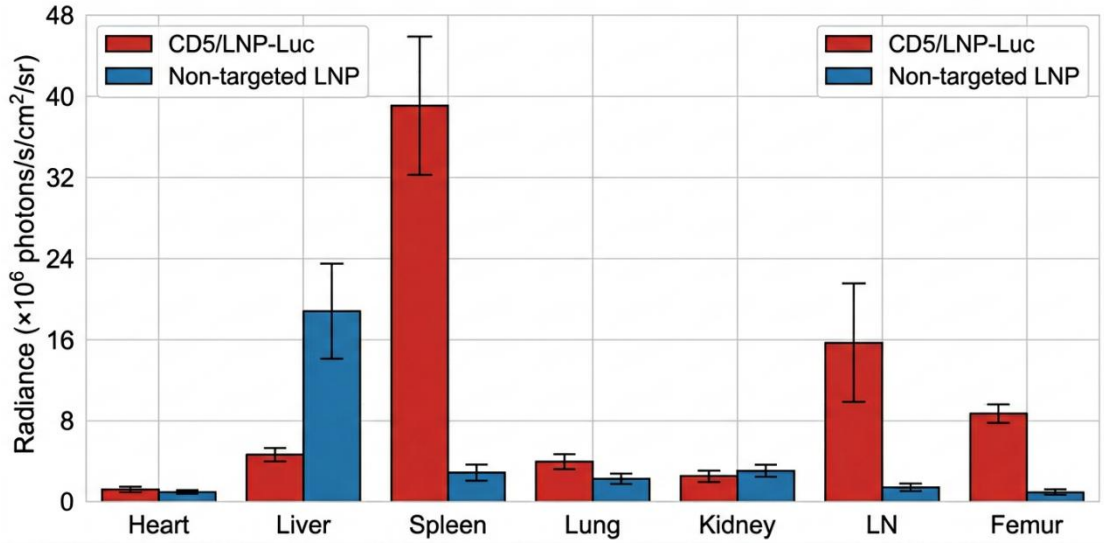

Figure S1: In vivo biodistribution (fluorescence quantification of isolated organs)

None of the treatment groups showed any aberrant conduct or noticeable weight loss during the monitoring period. Serum cytokine tests showed that IL-6 levels were 38.7 ± 8.2 pg/mL 24 hours after CD5/LNP-CAR19 was given. This was much lower than the positive control group (LPS-stimulated) at 1250 pg/mL. When compared to the model control group (28.4 ± 5.6 pg/mL), there was a little increase, although it was not statistically significant ($P = 0.29$) (Table 3).Furthermore, neither TNF-α nor IFN-γ levels showed any appreciable rise. The liver function markers ALT and AST, as well as the renal function marker creatinine, did not differ substantially across the groups. The absolute neutrophil and platelet counts in peripheral blood did not significantly decrease. None of the mice showed signs of neurotoxicity.

Table 3. Serum cytokine levels 24 hours after administration (pg/mL)

Cytokine	Normal control group	model control group	CD5/LNP-Luc	CD5/LNP-CAR19
IL-6	12.3 ± 3.2	28.4 ± 5.6	25.1 ± 4.7	38.7 ± 8.2
IFN-γ	8.1 ± 1.9	15.2 ± 3.8	13.9 ± 4.1	22.5 ± 6.3
TNF-α	5.6 ± 1.4	11.8 ± 2.7	10.3 ± 2.2	17.4 ± 4.9
IL-2	3.2 ± 0.8	6.7 ± 1.5	6.1 ± 1.3	9.8 ± 2.6

5.Discussion

This study demonstrated that CD5-targeted LNP-mRNA nanoparticles can directly instruct endogenous T cells in the body to express functional CD19 CAR instantaneously in an animal model of systemic lupus erythematosus for the first time. Deep B cell depletion and long-term illness remission result from this, and lymphocyte clearance is not required. By combining the safety benefits of transient mRNA expression, the established delivery platform of LNPs, and CD5-targeted T cell selectivity, this "in vivo ready-to-use" CAR-T strategy offers fresh experimental data and theoretical backing for transforming CAR-T therapy from a highly customised ex vivo engineered approach into a widely available, universal medication.

It has long been thought that successful expansion and long-term survival of adoptive CAR-T cells require fludarabine/cyclophosphamide lymphodepletion. The basic notion is that it releases homeostatic cytokines and eliminates competing endogenous lymphocytes. Nevertheless, this study demonstrates that a single injection of CD5/LNP-CAR19 causes CAR^+ T cells to peak at roughly 32% within 24 hours and drives over 95% depletion of peripheral and tissue B cells, with effects lasting more than 8 weeks, even in fully immune-competent MRL/lpr mice with no lymphodepletion at all.This casts doubt on the widely held notion that lymphodepletion is necessary for CAR-T therapy and implies that the behaviour of effector cells that are quickly programmed from endogenous T cells in the body differs significantly from that of long-lived CAR-T cells produced in a lab. Without requiring long-term CAR-T cell surveillance, the brief, focused, and self-limiting burst of cytotoxicity provided by the temporary expression window of mRNA (about 5 days) is sufficient to eliminate pathogenic B cell compartments.This result is consistent with clinical observations made by Mackensen and others in SLE patients: injected CAR-T cells rapidly proliferate and then swiftly contract even with conventional lymphodepletion, indicating that the important treatment window is during the early effector phase rather than protracted persistence. Therefore, the lymphodepletion-free approach created in this work more closely aligns with the actual requirements of treating autoimmune diseases: minimising treatment-related damage while restoring immunological homeostasis through deep but transient immune intervention.

This study extends that platform to the field of systemic autoimmune diseases for the first time and demonstrates that it can also efficiently kill B cells in circulation and lymphoid tissues without pre-conditioning, in contrast to the groundbreaking work by Rurik and others, who used CD5/LNP-mRNA to generate FAP-CAR-T cells in mice with cardiac fibrosis. In contrast to Rurik's target, activated cardiac fibroblasts, which are a localised target, $CD19^+$ B cells in SLE are extensively dispersed in bone marrow, lymph nodes, spleen, and peripheral blood, placing more demands on the distribution and effectiveness of CAR-T cells.In vivo programmed CAR-T cells naturally co-localize with B cells, which may be a crucial element in achieving profound depletion without pre-conditioning. Our biodistribution data demonstrate that CD5/LNP may effectively carry mRNA to T cell-rich regions including the spleen and lymph nodes. Additionally, mRNA's transient expression essentially eliminates insertional mutation risks compared to the tumorigenic risks from permanent integration by viral vectors; additionally, the in vivo programming strategy only requires a single intravenous injection, significantly reducing treatment barriers and costs and improving the accessibility of CAR-T therapy for non-malignant, high-prevalence diseases. This is in contrast to the complicated facilities and lengthy production times required for ex vivo culture.

It's interesting to note that the severe decrease of B cells persists for more than eight weeks, despite the fact that CAR mRNA is eliminated in five days. Survival rates increase from 40% to 90%, renal immune complex deposits practically vanish, urine protein recovers to normal, and anti-dsDNA antibodies decrease by about 80%.This "quick effect—long-term remission" asymmetry may be caused by a number of mechanisms: first, CD19 CAR-T cells eliminate the entire B cell lineage, from progenitor B cells to mature B cells, including some CD19-positive plasmablasts, so B cell recovery necessitates a slow process beginning from new bone marrow output; second, once the source of autoantibodies is cut off, existing immune complexes gradually degrade over weeks and complement consumption eases, which is consistent with the clinical observation of anti-dsDNA reduction and complement recovery; and third, complete B cell clearance may also disrupt the survival signals and antigen presentation support that autoreactive T cells.This outcome further confirms that deep CD19 clearance is adequate to accomplish immunological reset in SLE and is extremely congruent with the long-term remission trend shown by Kansal and others utilising ex vivo-prepared CD19 CAR-T cells in MRL/lpr mice.

Neurotoxicity, cytokine release syndrome (CRS), or severe bone marrow suppression were not noted in this investigation. Pro-inflammatory variables in serum, such as IL-6 and IFN-γ, fluctuated relatively slightly and did not vary statistically. Three design features may be responsible for this safety profile: first, CD5-targeted delivery restricts CAR expression to T cell compartments, preventing systemic type I interferon responses caused by non-specific transduction of antigen-presenting cells; second, the natural pharmacokinetics of mRNA limit the duration of CAR expression, preventing the cascade amplification of a "cytokine storm" caused by persistent T cell activation; and third, the absence of lymphodepletion preserves the body's intrinsic regulatory T cells and immune regulatory networks, which serve as an inherent buffer for the effector response. For SLE patients with various degrees of organ dysfunction, this safety benefit is particularly significant since it makes recurrent dosing or dose modifications much more clinically

feasible.

The results must be regarded cautiously because this study has certain limitations. First, while the spectrum of autoantibodies and nephritis observed in human lupus is fairly well replicated in the MRL/lpr model, the disease in these mice is dependent on Fas gene mutations, and the immune environment, LNP protein corona components, and pharmacokinetics differ from those in humans. Therefore, additional validation in large animal models, such as non-human primates, will be necessary to translate these findings to the clinic. Second, the duration of CARs is limited by the transitory expression of mRNA.The long-term risk of relapse is still unknown after B cells begin to recover without continuous effector control, and longer-term study of the post-recovery B cell phenotype (whether they are primarily naive or if autoreactive B cells resurface) is still necessary. Third, as CD5-targeted LNPs may activate complement or cause anti-drug antibodies, which may lessen the efficacy of subsequent treatments, repeated dosage may be restricted by possible anti-CAR or anti-LNP antibody reactions. Fourth, this approach does not entirely eradicate preexisting humoral immunological memory, and long-lived CD19-negative plasma cells are one of the persistent sources of autoantibodies in SLE. It is worthwhile to investigate whether combining with anti-plasma cell tactics (such as BCMA or CD38 targeting) may be required in the future. Lastly, a head-to-head comparison of the two approaches in the same model was not feasible because this study did not establish a parallel group using in vitro generated CD19 CAR-T cells. Future research should make that comparison.

To put it briefly, this study comprehensively showed for the first time that CD5-targeted LNP-mRNA can produce CD19 CAR-T cells in vivo on site, safely and successfully causing long-term remission in lupus models without lymphodepletion. This tactic is a crucial step in transforming CAR-T therapy from a "personalised cell drug" into a "off-the-shelf universal formulation" by moving the primary stage of CAR-T creation from outside the body to inside. Future studies should concentrate on improving the LNP formulation's cell specificity and transfection efficiency, elucidating the precise dynamics and functional characteristics of immune reconstitution following B cell depletion, assessing the impact of repeated dosing on the maintenance of long-term remission, and confirming the safety and efficacy of this strategy in large animal autoimmune models that are more similar to clinical settings. This will ultimately open the door for its application to human SLE and other B cell-mediated autoimmune diseases.

6.Conclusion

Using lipid nanoparticles conjugated with CD5 antibodies to carry mRNA encoding the CD19 chimeric antigen receptor (CD5/LNP-CAR19), this study is the first to propose and systematically validate a "in vivo ready-to-use" CAR-T therapy strategy. This approach can directly program endogenous T cells in MRL/lpr lupus mice to express functional CARs instantly through a single intravenous injection, all without the need for lymphodepletion beforehand. The following are the key conclusions:

Effective programming of T cells in vivo: The percentage of CAR^+ T cells in peripheral blood reached 32.4% just 24 hours after a single injection, and lymphoid organs like the spleen, lymph nodes, and bone marrow all demonstrated targeted delivery and expression, demonstrating that the CD5/LNP system can effectively produce CAR-T cells in the body. Deep and long-lasting B cell depletion: A single treatment caused over 95% B cell depletion in peripheral blood and tissues, even in the absence of lymphocyte clearance. This effect persisted for more than eight weeks, demonstrating that a short window of mRNA expression is sufficient to promote complete and long-lasting target cell clearance.Significant disease remission and survival benefit: Serum anti-dsDNA autoantibodies decreased by 78.4% in the treated group, urine protein/creatinine ratios returned to normal, kidney immune complex deposits and pathological damage were nearly entirely reversed, survival rates rose from 40% to 90%, and no serious side effects were noted. Excellent safety profile: Key pro-inflammatory cytokine levels did not rise noticeably, demonstrating the strong controllability of this technique. No cytokine release syndrome, neurotoxicity, or bone marrow suppression were seen throughout.

It essentially avoids viruses from merging into viral vector and lymphodepletion toxicity, customised manufacturing of CAR-T production from outside to inside, extends the treatment from “personalised cell transplantation” to “off-the-shelf universal therapy”, and preserves the essential mechanism of severe CD19 depletion. It can be used to optimize LNP formulations and ligands to further improve transfection efficiency and T cells subset selection; to assess the effect of repeated doses on anti-drug immune response and long-term relapse control; to test pharmacokinetics,

efficacy and safety in non-human primate immune system models, and to investigate the potential of its platform for other B cell-based autoimmune disease such as pemphigus vulgaris, myasthenia gravis, and systemic sclerosis, which could help to revolutionize cell therapy for autoimmune diseases and provide more patients with a new, safe, and open treatment option.

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